# BIOTRANSFORMATION OF NITROAROMATIC COMPOUNDS BY METHANOGENIC ARCHAEA

FINAL PROGRESS REPORT

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13. ABSTRACT *(Maximum 200 words)*

The specific aim of this proposal has been to clarify some of the discrepancies in the literature regarding nitroaromatic transformation by methanogenic archaebacteria. We have shown that methanogens are able to transform only a small number of nitroaromatics including *p*-nitrophenol, *m*-nitrophenol, 2,4-nitrophenol, *p*-nitrobenzoic acid, and *p*-nitroaniline, but not 2,4,6-trinitrotoluene or 2,6-dinitrotoluene. In all cases the methanogens lyse unless they are pregrown to a know optical density before adding the particular nitroaromatic compound.

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Enclosure 1

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## 1. Foreword

The pollution of soil and water with explosives and related compounds caused by military activities has been known for a long time, but progress in understanding the environmental fate of such substances has only been made in the last few years. Many of these environmental are anaerobic in nature and microbial processes could be used for the remediation of explosive-contaminated soils and waste waters. The use of anaerobic microorganisms or enzymes in the biocatalytic production industrially valuable products from nitroaromatics also immediately comes to mind, however, very little is known about the enzymatic mechanisms of the organism that degrade these compounds. It was the overall goal of our research to define a logical path by which we may begin to define these mechanisms and eventually manipulate the enzymes involved.

## 2. Technical Report

The methanogenic archaea have been previously shown to convert a number of nitroaromatic compounds to their corresponding, more toxic, nitroamines. Our research proposal was originally based on these reports. From our work, we have discovered a number of disturbing discrepancies between the original work and ours. We have spend the last three years attempting to clarify these issues and have had limited success in repeating the published work. Interestingly, since the time of our proposal, there have been only three reports of transformation of nitroaromatics by methanogens and in all cases limited success was reported

Our work focused on two compounds, 2,4,6-trinitrotoluene (TNT) and 2,6-dinitrotoluene (2,6-DNT) is a predicted breakdown product of TNT. TNT is an explosive widely used in the military because of its characteristics such as a low melting point, stability, low sensitivity to impact, friction, and high temperature, and its relative safe methods of manufacture. Soil and water have been extensively contaminated owing to manufacturing and testing of explosives. White rot fungus has been shown to mineralize radiolabeled TNT (4). Under anaerobic conditions a sulfate-reducing bacterium, *Desulfovibrio* sp. (B strain), transformed TNT to toluene (1) by reduction followed by a reductive deamination reaction. Boopathy and Kulpa (2) reported the biotransformation TNT by a *Methanococcus* sp. (strain B) isolated from a lake sediment. Unfortunately this species was lost upon storage.

We have looked at three strains of methanogens for their ability to degrade the two nitroaromatics above. *Methanococcus thermolithotrophicus* is a autotrophic thermophilic methanogen isolated from geothermally heated sea sediments. Growth occurs on $H_2/CO_2$ at an optimum temperature of 65°C. *Methanosarcina thermophila* and *Methanosarcina barkeri* are two acetate-utilizing methanogens. It was our hypothesis early on that methanogens that utilize different substrates may exhibit different patterns of nitroaromatic metabolism.

The literature reported that one can simply add TNT to an exponentially-growing

methanogen culture and transformation will begin immediately. This is now clearly not the case as we have previously reported in former technical reports. *M. thermolithotrophicus* needs to be adapted at very low levels (10 µM) of either TNT or 2,6-DNT before growth will ensue. After adaptation, growth rates are comparable to that of cells in the absence of nitroaromatic. We believe that in fact we may be selecting for mutants which have adapted to the low levels of nitroaromatics. Continuous passage of the cultures through a number of inoculations results in the loss of the ability to survive in the presence of the nitroaromatic compounds. While it is difficult to culture these organisms on solid media, we have checked for plasmids in an adapted culture using standard plasmid isolation procedures. No plasmids were found in *M. thermolithotrophicus* suggesting other mechanisms for resistance to this compound. Nitroaromatics were clearly very toxic to this strain of methanogen. Since methanogens rely almost entirely on membrane potential ($H^+$ and $Na^+$ gradients) to obtain energy during their metabolism, the accumulation of apolar pollutants in bacterial membranes probably causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis. Microscopic observation of *M. thermolithotrophicus* confirms this hypothesis; exponentially-growing cells lyse within two hours of exposure to either nitroaromatic compound. Our early reports of adaptation to 0.5 mM 2,6-DNT suggest that we had a mutant and subsequently lost it upon subculturing. The acetate-utilizing methanogenic strains, *M. thermophila* and *M. barkeri* show similar results.

To determine if the substituents on the nitroaromatic ring were involved in the inhibition of growth of the acetate-utilizing methanogens, we investigated the ability of three strains (*M. thermophila*, *M. barkeri*, and *M. frisia*) to transform several nitroaromatic compounds including *p*-nitrophenol (*p*-NP), *m*-nitrophenol (*p*-MP), 2,4-dinitrophenol (2,4-DNP), *p*-nitrobenzoic acid (*p*-NB), and *p*-nitroaniline (*p*-NA). Results are shown in the table below.

| | Nitroaromatic compound* | | | | |
|---|---|---|---|---|---|
| Organism | *p*-NP | *m*-NP | 2,4-DNP | *p*-NB | *p*-NA |
| *Methanosarcina thermophila* | + | + | + | + | + |
| *Methanosarcina barkeri* | + | + | + | + | + |
| *Methanosarcina frisia* | + | + | + | + | + |

*all compounds tested at 50 µM. All cells were grown to an optical density of 0.15 in typical media at 37°C except for *M. thermophila* which was grown at 50°C before addition of the nitroaromatic

Like *M. thermolithotrophicus*, the transformation of nitroaromatic compounds by the acetate-utilizing methanogens only occurred while cells lysed. It was necessary to pregrow the cells to an optical density of about 0.15 before adding the nitroaromatics. Clearly all the methanogens tested, as shown in the table above, were able to perform a transformation of the nitroaromatics. Cell lysis did not depend on the type of nitroaromatic compound. This may be the reason why methanogenesis is inhibited by nitroaromatics during anaerobic sewage treatment (3). Therefore, we may be able to assume that the nitroaromatics or intermediates of the reduction process like nitroso- or hydroxylamines are the real toxicants, since they may react with the unique cell membrane components of the methanogens. A second toxic function of the nitroaromatic compound might be determined by the fact that it acts as an 'electron trap'. This has already been assumed for the oxygen sensitivity of methanogens (5), consequently leading to the breakdown of ATP synthesis. A third possibility might be the uncoupling activity of nitroaromatics on electron transport chains thus inhibiting ATP synthesis, followed by cell lysis. Further investigation was done to determine if reducing agents could abiotically transform the compounds tested. Two commonly used reducing agents used in methanogenic media are sulfide and cysteine. Sulfide was not able to reduce *p*-NP, *m*-NP, or *p*-NB but could partially reduce 2,4-DNP, and *p*-NA. Cysteine showed the same pattern of reduction. Further investigation into the degradation of *p*-NP is currently being done since it is apparently not abiotically reduced.

## 3. Supported personnel

Mr. Chad Pearion was supported by this grant. He is currently pursuing his Ph.D. degree and has passed his written exams.

## 4. Bibliography


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